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Ferrari, Giada, Cuevas, Angélica, Gondek-Wyrozemska, Agata T. et al. (15 more authors) (2021) The preservation of ancient DNA in archaeological fish bone. Journal of archaeological science. 105317. ISSN 0305-4403

https://doi.org/10.1016/j.jas.2020.105317

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Journal of Archaeological Science 126 (2021) 105317

Contents lists available at ScienceDirect

Journal of Archaeological Science

journal homepage: http://www.elsevier.com/locate/jas

The preservation of ancient DNA in archaeological fish bone

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ARTICLE INFO

Keywords:
Endogenous DNA
Bleach
Bone element
Bone remodeling
Petrous bone

ABSTRACT

The field of ancient DNA is dominated by studies focusing on terrestrial vertebrates. This taxonomic bias limits our understanding of endogenous DNA preservation for species with different bone physiology, such as teleost fish. Teleost bone is typically brittle, porous, lightweight, and is characterized by a lack of bone remodeling during growth. All of these factors potentially affect DNA preservation. Using high-throughput shotgun sequencing, we here investigate the preservation of DNA in a range of different bone elements from over 200 archaeological Atlantic cod (*Gadus morhua*) specimens from 38 sites in northern Europe, dating up to 8000 years before present. We observe that the majority of archaeological sites (79%) yield endogenous DNA, with 40% of sites providing samples containing high levels (>20%). Library preparation success and levels of endogenous DNA depend mainly on excavation site and pre-extraction laboratory treatment. The use of pre-extraction treatments lowers the rate of libraries that can be sequenced, although — if successful — the fraction of endogenous DNA can be improved by several orders of magnitude. This trade-off between library preparation success and levels of endogenous DNA allows for alternative extraction strategies depending on the requirements of down-stream analyses and research questions. Finally, we do not find particular bone elements to yield higher levels of endogenous DNA, as is the case for denser bones in mammals. Our results highlight the potential of archaeological fish bone as a source for ancient DNA and suggest a possible role of bone remodeling in the preservation of endogenous DNA.

1. Introduction

Driven by revolutionary advances in laboratory methods, sequencing technologies and computational analyses, an increasing number of terrestrial vertebrate species have been investigated using ancient DNA (aDNA). Such studies have addressed a wide range of questions related to, for example, extinct megafauna, animal domestication, or archaic human history (e.g., Hofreiter et al., 2015; Ollivier et al., 2018;

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https://doi.org/10.1016/j.jas.2020.105317
Received 27 August 2020; Received in revised form 27 November 2020; Accepted 21 December 2020
Available online 9 January 2021
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Palkopoulou et al., 2018; Skoglund and Mathieson, 2018) and have yielded fundamental methodological insights. For instance, a seminal discovery revealed that the petrous bone, i.e., the *pars petrosa* of the temporal bone, which is the hardest and densest bone in mammals (Frisch et al., 1998), has an increased potential of containing high levels of endogenous DNA (Gamba et al., 2014; Pinhasi et al., 2015). Knowing which type of bone element may yield the best results for aDNA research is crucial for a variety of reasons. First, focusing on sampling bone elements with high endogenous DNA greatly improves the economy of high-throughput sequencing studies (Rizzi et al., 2012) by lowering the cost per sequenced base, thus helping to avoid costly analyses for samples that are likely suboptimal. Second, sampling for aDNA is most often destructive. Knowing how to select the right elements helps minimize the destruction of unique archaeological materials that represent a finite resource (Pálsdóttir et al., 2019). Third, such knowledge may further aid archaeologists in making informed choices when collecting and preserving zooarchaeological material in the field, maximizing the research potential for a variety of studies. This insight has therefore transformed the field of aDNA, allowing the cost-efficient, genome-wide analysis of hundreds of individual ancient specimens (e.g., Damgaard et al., 2018; Fages et al., 2019; Mathieson et al., 2018; Olalde et al., 2018).

In mammals, low bone density is usually associated with poor DNA preservation (Geigl and Grange, 2018). Archaeological fish bone (Fig. 1A) is typically lightweight, porous, brittle and susceptible to taphonomic damage (Szpak, 2011) and such bone could thus be considered a suboptimal source of aDNA from a mammalian preservation perspective. In contrast to mammals, however, fish bone does not serve as a calcium reservoir under normal conditions (Moss, 1961; Witten and Huysseune, 2009). Most higher teleosts, including Atlantic cod (*Gadus morhua*), lack osteocytes (Kranenbarg et al., 2005; Moss, 1961; Shahar and Dean, 2013; Witten and Villwock, 1997). In these fish with acellular bone, bone remodeling takes place to a lesser extent and through different cellular and physiological processes (Harland and Van Neer, 2018; Kranenbarg et al., 2005; Witten and Villwock, 1997). An absence of bone remodeling may be important for DNA preservation for several reasons. For example, it has been suggested that an absence of cell *lacunae* improves the resistance of acellular fish bone to microbial degradation (Szpak, 2011). Moreover, recent evidence indicates that an absence of bone remodeling may aid DNA preservation in specific mammalian bone elements (Kontopoulos et al., 2019). It is therefore possible that the fundamental differences between mammalian and fish skeletal physiology, and especially the lack of bone remodeling in most fish, affects the aDNA preservation potential of archaeological fish bone.

Interestingly, multiple studies have reported the successful retrieval of aDNA from archaeological fish bone for a variety of species, locations and age (Oosting et al., 2019). Ancient DNA has been successfully amplified from Pacific herring (*Clupea pallasi*, Speller et al., 2012; Moss et al., 2016), Atlantic salmon (*Salmo salar*, Royle et al., 2020), Pacific salmon (*Oncorhynchus* spp, Grier et al., 2013; Johnson et al., 2018; Royle et al., 2018; Speller et al., 2005; Yang et al., 2004), Atlantic cod (*G. moruha*, Hutchinson et al., 2015; Ólafsdóttir et al., 2014), sturgeon (*Acipenser* spp., Ludwig et al., 2009; Nikulina and Schmölcke, 2016; Pagès et al., 2009), trout species (*Salmo trutta*, Splendiani et al., 2016; *Salvelinus namaycush*, Royle et al., 2020), Northern pike (*Esox lucius*, Wooller et al., 2015), and other fish taxa (*Clarias gariepinus*, Arndt et al., 2003; *Abramis brama*, Ciesielski and Makowiecki, 2005; *Rutilus frisii*, Živaljević et al., 2017), in some cases from bones up to 6000 yBP or older (Johnson et al., 2018; Nikulina and Schmölcke, 2016; Speller et al., 2012; Moss et al., 2016; Splendiani et al., 2016; Wooller et al., 2015; Yang et al., 2004). Fish aDNA has also been successfully amplified in metagenomic analyses using bulk bone approaches (Seersholm et al., 2018), even from warm tropical climates (Grealy et al., 2016). Finally, high-throughput sequencing (HTS) approaches have yielded high levels (15–50%) of endogenous DNA from a limited number of sites up to one thousand years old (Boessenkool et al., 2017; Star et al., 2017). These studies range from species identification (e.g., Yang et al., 2004; Ludwig et al., 2009), to more complex reconstructions of past population diversity and demographic events (e.g., Ólafsdóttir et al., 2014; Johnson et al., 2018). In addition to addressing biological questions, fish aDNA also provides insight into historic and prehistoric use of fish by humans (e.g., Grier et al., 2013; Speller et al., 2005) and their trading routes (e.g., Arndt et al., 2003; Star et al., 2017). Despite the clear potential for

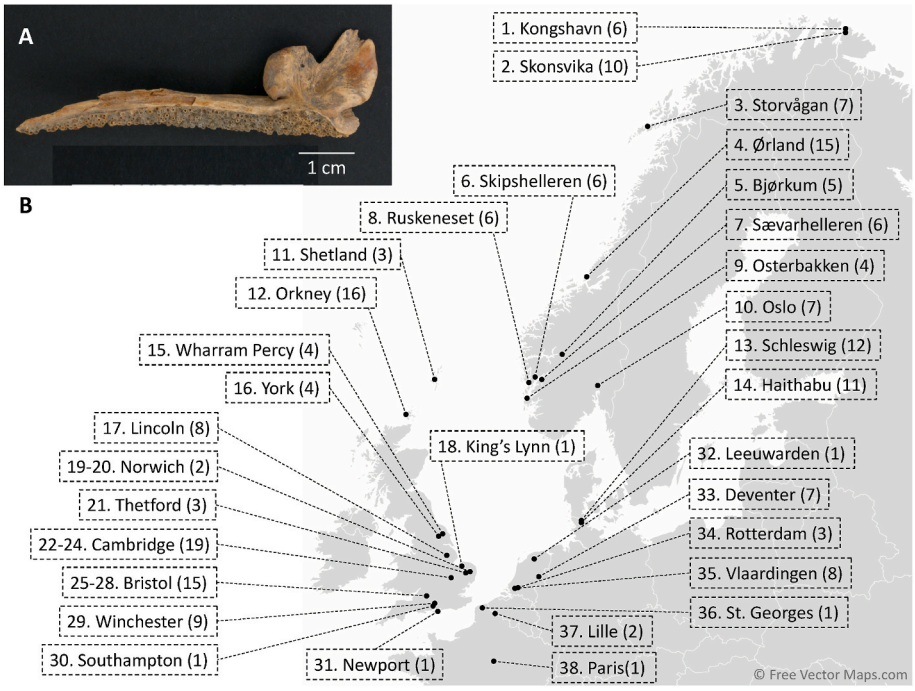

Fig. 1. Archaeological Atlantic cod bones. (A) Archaeological Atlantic cod jawbone (premaxilla) from the site of Orkney Quoygrew (1000–1200 CE). (B) Locations of fish bone specimens (n = 204) from 38 archaeological sites. Bones from Norwich, Cambridge and Bristol were obtained from distinct, individually numbered archaeological sites (see Table 1).

aDNA preservation in archaeological fish remains, however, no studies have yet investigated the factors that underlie this preservation and it is unclear if the expectation of intra-skeletal variability in DNA preservation observed for mammals is applicable to other vertebrate taxa such as fish.

Here, we investigate the preservation of aDNA in archaeological Atlantic cod bones ($n = 204$) obtained from 38 excavations in northern Europe, dating from 6500 BCE to c.1650 CE (spanning the Mesolithic to early modern periods, Fig. 1B, Tables 1 and S1). We use a HTS approach to investigate whether bone element, archaeological site, DNA extraction method, and/or sequencing library preparation protocol can be used to predict library success (i.e., the sufficient retrieval and amplification of aDNA for HTS sequencing) and the relative proportion of endogenous DNA. We interpret our results in light of down-stream analytical requirements and provide practical recommendations in order to maximize throughput and archaeological inference of whole genome sequencing (WGS) data from ancient fish bone.

2. Materials and methods

2.1. Sample processing and DNA extraction

A total of 204 Atlantic cod bones originating from 38 sites (Fig. 1B, Tables 1, S1 and S2) were processed following one of three DNA extraction protocols: (1) standard extraction (adapted from Dabney et al., 2013), (2) with the inclusion of a pre-digestion step (DD, Damgaard et al., 2015), or (3) with the addition of a mild bleach treatment and pre-digestion step (BLEDD, Boessenkool et al., 2017). All laboratory protocols were carried out in a dedicated aDNA clean laboratory at the University of Oslo following standard anti-contamination and authentication protocols (e.g., Cooper and Poinar, 2000; Gilbert et al., 2005; Llamas et al., 2017). Bones were UV-treated for 10 min per side and pulverized using a stainless-steel mortar (Gondek et al., 2018) or a Retsch MM400 mixer mill. Up to two times 150–200 mg of bone powder was digested for 18–24 h in 1 ml 0.5 M EDTA, 0.5 mg/ml proteinase K and 0.5% N-Laurylsarcosine. Digests were combined and DNA was extracted with 9 × volumes of PB buffer (QIAGEN) or a 3:2 mixture of QG buffer (QIAGEN) and isopropanol. MinElute purification was carried out using the QIAvac 24 Plus vacuum manifold system (QIAGEN) in a final elution volume of 65 μl. Parallel non-template controls were included. A subset of 73 samples was subjected to multiple treatments (Table S1).

2.2. Library preparation, sequencing and read processing

Single- or double-indexed blunt-end sequencing libraries were built from 15 to 16 μl of DNA extract or non-template extraction blank, following either the single-tube (BEST) protocol (Carøe et al., 2018) with the modifications described in Mak et al., (2017) or following the Meyer-Kircher protocol (Kircher et al., 2012; Meyer and Kircher, 2010) with the modifications listed in Schroeder et al., (2015). Blunt-end repair, adapter ligation and set up of indexing PCRs were performed in the aDNA clean laboratory. Library quality and concentration were inspected with a High Sensitivity DNA Assay on the Bioanalyzer 2100

Table 1
Archaeological sites ($n = 38$) in northwest Europe from which archaeological Atlantic cod bones were obtained. For each site, the country, date and number of bones (n) are provided. Dating is based on archaeological context. For locations see also Fig. 1B. NO = Norway, UK = United Kingdom, DE = Germany, NL = The Netherlands, F = France.

ID	Site name	Country	Date (BCE/CE)	Samples (n)	References
1	Kongshavn	NO	1300-1400 CE	6	Amundsen (2011)
2	Skonsvika	NO	1240-1390 CE	10	Amundsen (2011)
3	Storvågan	NO	1150-1270 CE	7	Wickler (2013)
4	Ørland Main Air Base	NO	200-400 CE	15	Storå et al. (2019)
5	Bjørkum	NO	700-1000 CE	5	(Barrett et al., 2015); M. Ramstad, pers. comm)
6	Skipshelleren	NO	6000 BCE - 1000 CE	6	(Hjelle et al., 2006; Rosvold et al., 2013)
7	Sævarhelleren	NO	6500-6200 BCE	6	Bergsvik et al. (2016)
8	Ruskeneset	NO	2000 BCE - 1000 CE	6	Hufthammer (2015)
9	Osterbakken	NO	2000 BCE - 1000 CE	4	Hufthammer (2015)
10	Oslo Mindets tomt	NO	pre-1175-1350 CE	7	Lie (1988)
11	Shetland Sandwick South	UK	1100-1350 CE	3	Bigelow (1989)
12	Orkney Quoygrew	UK	1000-1200 CE	16	Harland & Barrett (2012)
13	Schleswig Schild	DE	c.1050–1280 CE	12	Heinrich (1987)
14	Haithabu Harbour	DE	c.800–1050 CE	11	Heinrich (2006)
15	Wharram Percy	UK	1250-1400 CE	4	Barrett (2005)
16	York Coppergate 16-22	UK	1040-1375 CE	4	Harland et al. (2016)
17	Lincoln Castle	UK	1150-1200 CE	8	(Barrett, unpublished)
18	King's Lynn Raynham House	UK	1250-1350 CE	1	Locker (2000)
19	Norwich Castle Mall	UK	1050-1100 CE	1	Locker (2009)
20	Norwich Fishergate	UK	1000-1150 CE	1	Locker (1994)
21	Thetford St. Barnabas' Hospital	UK	c.1000–1100 CE	3	Jones (1984)
22	Cambridge Corpus Christi College	UK	1500-1600 CE	4	Harland (2007)
23	Cambridge Grand Arcade	UK	1300-1600 CE	12	Harland (2019)
24	Cambridge St. John's Triangle	UK	1550-1650 CE	3	Harland (2009)
25	Bristol Broad Quay	UK	c.1000–1200 CE	1	Russ (2011)
26	Bristol Dundas Wharf	UK	c.1225–1400 CE	2	Jones & Watson (1987)
27	Bristol Finzel's Reach	UK	1125-1500 CE	9	Nicholson (2017)
28	Bristol Redcliff Street 82-90	UK	c.1125–1375 CE	3	Nicholson (2000)
29	Winchester Brook Street	UK	1000-1350 CE	9	(Barrett, unpublished)
30	Southampton French Quarter	UK	1250-1350 CE	1	Nicholson (2011)
31	Newport Ship	UK	c.1469 CE	1	Russ (2012)
32	Leeuwarden Oldehoofsterkerkhof	NL	725-900 CE	1	Thilderkvist (2013)
33	Deventer Burseplein 434	NL	1100-1250 CE	7	Beerenhout (2015)
34	Rotterdam Hoogstraat	NL	1300-1400 CE	3	Carmiggelt et al. (1997)
35	Vlaardingen Gat in de Markt	NL	1200-1350 CE	8	Buitenhuis et al. (2006)
36	St. Georges sur l'Aa	F	800-1000 CE	1	Clavel et al. (2015)
37	Lille Chateau de Courtrai	F	1300–1400	2	Clavel (2001)
38	Paris St. Michel	F	1400-1500 CE	1	Clavel (2001)

(Agilent) or with a High Sensitivity NGS Fragment Analysis Kit on the Fragment Analyzer™ (Advanced Analytical). Libraries with a minimum concentration of 0.1 ng/μl and with an average insert size of 25–70 bp were sequenced on the Illumina HiSeq 2500 or HiSeq 4000 platforms at the Norwegian Sequencing Centre with paired-end 125 bp (HiSeq 2500) or 150 bp (HiSeq 4000) reads and demultiplexed allowing zero mismatches in the index tag. Libraries with more than 100,000 reads were downsampled (n = 100,000) and processed using PALEOMIX v.1.2.13 (Schubert et al., 2014). Paired-end reads were trimmed, filtered, and collapsed with AdapterRemoval v.2.1.7 (Lindgreen, 2012), discarding reads shorter than 25 bp. Collapsed reads were aligned to the most recent version of the Atlantic cod reference genome, gadMor3 (RefSeq accession GCF_902167405.1, Star et al., 2011; Tørresen et al., 2017) with BWA v.0.7.12 (Li and Durbin, 2009), using the aln algorithm with disabled seeding. Endogenous DNA content was defined as the unique fraction of reads (corrected for clonality using PicardTools) with a minimum quality score of 25. Ancient DNA deamination patterns were assessed with mapDamage v.2.0.6 (Jónsson et al., 2013).

2.3. Statistical analysis

The data presented here are resulting from a long-term research project lasting several years. For various reasons, sample treatments are not evenly represented, reflecting for instance, temporal updates or alterations to protocols based on earlier results (Tables S1 and S2). We considered two metrics of "success": (1) the generation of sequencing libraries with sufficient concentration for HTS (0.1 ng/μl) that yielded a minimum of 10′000 sequencing reads and (2) levels of endogenous DNA in successfully sequenced libraries. Samples that underwent multiple treatments (n = 73, 146 treatments) were used to fit a Generalized Linear Mixed Effect Model (GLM, family = binomial, using sample ID as random effect to account for paired data) to test the effect of DNA extraction protocol, library preparation protocol, site, and bone element on failure or success of library preparation (library outcome ~ extraction protocol + library protocol + site + bone element + (1 | Sample)). Outcome of library preparation was also assessed using all generated libraries, excluding sites with less than three samples (n = 191) and controlling for multiple treatments by randomly subsampling one treatment per sample. Subsampling was performed 100 times generating (i = *100*) resampled datasets. A GLM (library outcome ~ extraction protocol + library protocol + site + bone element) was run on all resampled datasets. A sensitivity analysis was run to evaluate the consistency of the results recording significant factors for each iteration. In order to test the effect of DNA extraction protocol, library preparation protocol, site, and bone element on endogenous DNA content, successfully sequenced libraries (defined as libraries that yielded more than 10,000 sequencing reads), excluding sites with less than three successful libraries (n = 124 from 19 sites), were used to fit a Generalized Linear Regression (GLR, endogenous DNA fraction ~ extraction protocol + library protocol + site + bone element). Normality of the data for endogenous DNA content was tested by levels in each of the factors using a Shapiro-Wilk Normality Test. For the GLM and GLR described above, several models were run discarding factors that did not show significance in more complex models. Akaike (AIC) and Bayesian Information Criterion (BIC) were used to select the best fitting models.

3. Results

A total of 277 sequencing libraries were generated from 204 Atlantic cod bones collected at 38 archaeological sites (Fig. 1B, Tables S1 and S2). Of these, 140 libraries from 29 sites had a minimum concentration of 0.1 ng/μl and were sequenced to a minimum of 10′000 reads (Figs. 2A and S1). All libraries showed patterns of DNA fragmentation, fragment length, and deamination rates that were consistent with those of authentic aDNA (Jónsson et al., 2013; Figs. S1 and S2, Table S1). Most samples (n = 131) were processed once, but a subset of samples (n = 73) were processed using two or more treatment combinations, either using different extraction or library preparation protocols (Fig. 2B, Table S1). Bone elements were categorized into three major groups – cranial, postcranial, and pectoral girdle bones (Fig. 3A). The representation of these major groups differs across sites (Fig. 3B, Table S1), which is driven by the availability of elements at the different locations or by post-excavation sample selection (Box 1).

Library preparation following the standard extraction protocol generated more successful sequencing libraries (70 out of 84 libraries yielded more than 10,000 sequencing reads) compared to the double digestion (DD) or combined double digestion and bleach (BLEDD) protocols (7 of 26 libraries for DD and 62 of 167 libraries for BLEDD, Fig. 2A). The Meyer-Kircher (MK) library preparation protocol yielded a higher success rate (107 out of 150 libraries) than the single tube library protocol (BEST) (32 out of 127 libraries, Fig. 2A). A number of library preparations (n = 62) for sites with initial high failure rates were repeated using the standard extraction protocol without pre-extraction washes (Fig. 2B) resulting in greater success rates. For example, library preparations after DNA extraction using the DD (n = 4) or BLEDD (n = 5) protocols for samples from the site of Ørland Main Air Base (site 4) failed, while library preparation following the standard DNA extraction protocol was more successful (14 out of 17 libraries, Table S1).

To statistically infer the most important factors explaining library success we applied two models. First, we focused on the samples that were processed with multiple treatments (n = 73, Fig. 2B). Second, we incorporated all samples generated from sites with more than three samples (n = 191 samples from 27 sites), correcting for multiple treatments by randomly downsampling a single treatment per sample iteratively (i = 100, Fig. 2C). The GLM focusing on the samples with multiple treatments (library outcome ~ extraction protocol + library protocol + site + bone element + (1 | Sample)) shows that the outcome is significantly dependent on DNA extraction and library preparation protocols (Table S3). The sensitivity analysis with the 100 iterations of GLMs incorporating all samples (library outcome ~ extraction protocol + library protocol + site + bone element) shows similar results, with site and DNA extraction protocol as the most prevalent significant factors (presenting mean estimates across iterations of 4.50 and 3.79 respectively, Fig. 2C), followed by library preparation protocol (mean estimate = 2.90). Bone element has no significant effect on library preparation outcome and after excluding it from the model the latter shows a better fit to the data (Table S4).

We further assessed whether the same factors affect levels of endogenous DNA for samples (n = 124) from 19 locations for which three or more specimens were successfully sequenced by fitting a GLR (endogenous DNA fraction ~ extraction protocol + library protocol + site + bone element). Significantly higher endogenous DNA contents are observed in samples that underwent the DD or BLEDD pre-treatments, compared to a standard DNA extraction (Fig. 4A, Table S5). Given that a number of samples for which DD or BLEDD extraction failed (n = 62) were re-extracted using the standard protocol (Fig. 2B), such samples may *a priori* be suspected to have relatively poor DNA preservation. In contrast, library preparation protocol had no significant effect on endogenous DNA content (Table S5). Although postcranial bones tend to have lower levels of endogenous DNA, these differences are not significant, and especially bones from the cranial and pectoral girdle yield comparable levels of endogenous DNA, independent of DNA extraction protocol (Fig. 4B, Table S5). Finally, we observe significant differences in endogenous DNA between sites (Fig. 4C, Table S5) with 8 out of 19 sites yielding samples with high levels (>20%) of endogenous DNA, which includes the oldest excavation (Sævarhelleren, site 7, dated to ca. 6500-6200 BCE). When excluding the non-significant factors from the GLR (bone element and library preparation protocol, Table S6), DNA extraction protocol and site remain significant. The most complex model including all factors shows the best fit to the data (Table S6).

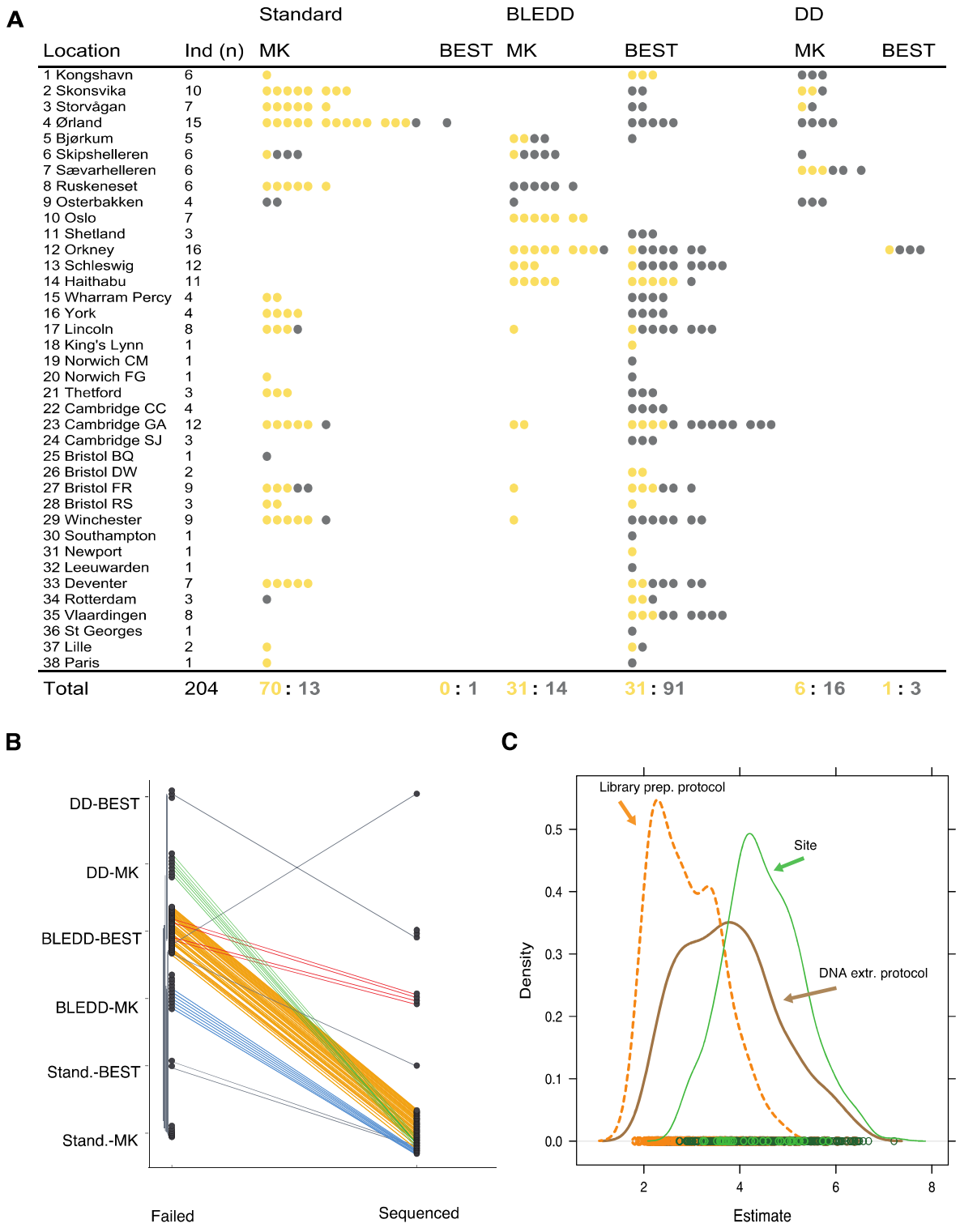

Fig. 2. Success rates of high-throughput library preparation from Atlantic cod bones. (A) Schematic of libraries generated for all archaeological sites (yellow, sequenced; gray, processed but not sequenced library) divided into groups according to DNA extraction and library preparation protocols utilized. Note that the number of libraries can be higher than the number of specimens (Ind) due to multiple treatments. DD = double digestion extraction protocol, BLEDD = bleach treatment combined with double digestion extraction protocol, MK = Meyer-Kircher library preparation protocol, BEST = single tube library preparation protocol. (B) Treatment overview for samples processed using multiple library and extraction treatment combinations (n = 73). Treatments are abbreviated according to Fig. 2A, and different treatment combinations per sample are indicated by connecting lines and colored according to treatment combination. (C) Sensitivity analysis: density distribution of significant factors (site, DNA extraction and library preparation protocols) following iterative ($i = 100$) logistic regression (library outcome ~ extraction protocol + library protocol + site + bone element + (1 | Sample)), using randomly resampled data. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

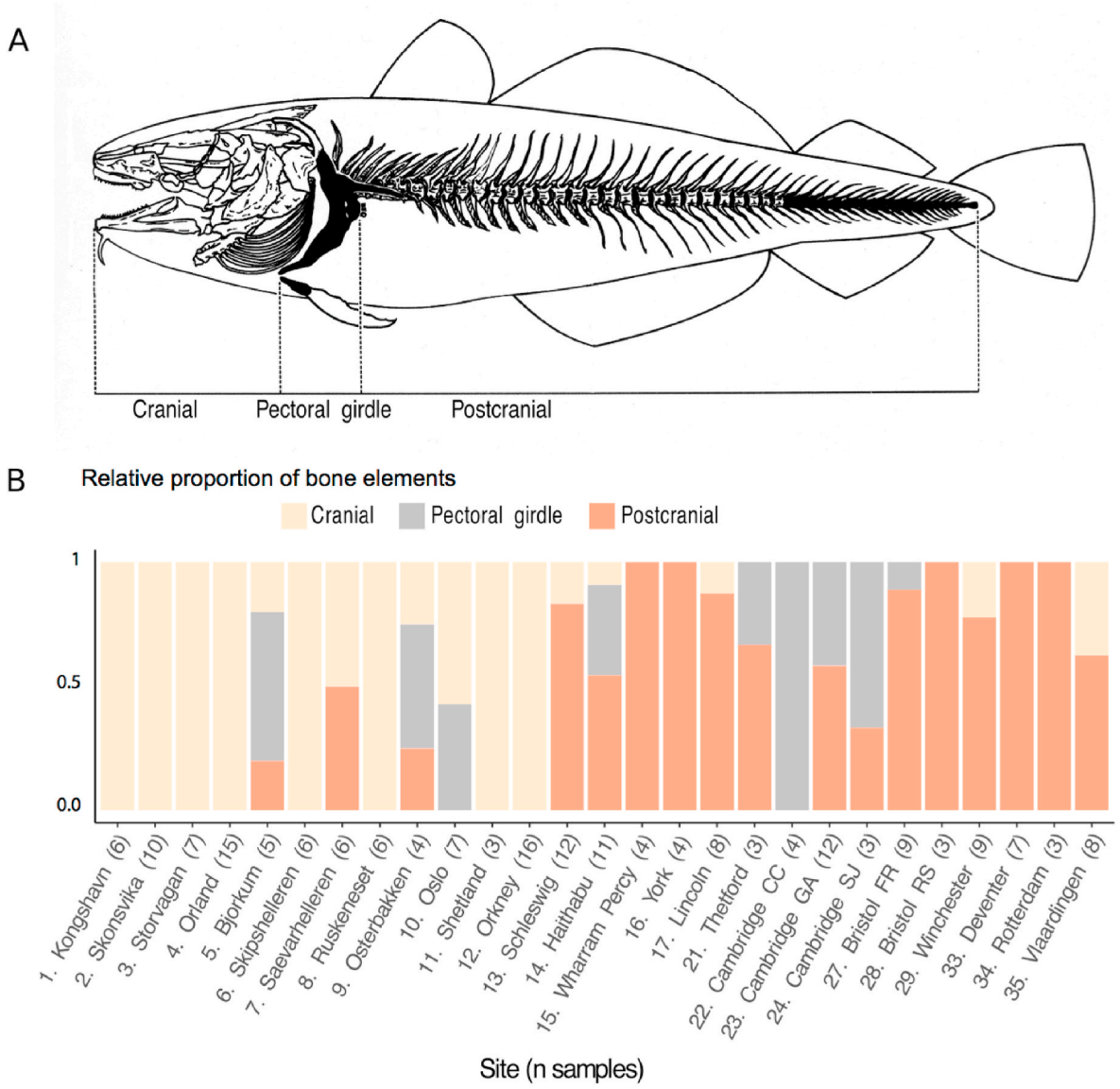

Fig. 3. Distribution of Atlantic cod bone elements. (A) Classification of fish bone element groups. Adapted from Barrett et al., (1999). (B) Distribution of bone elements groups per site. Only sites with three or more samples ($n = 27$) are shown. Note that the distribution of selected bone elements is not necessarily representative of their relative rate of retrieval at specific sites.

Box 1

As part of the conservation process prior to long-distance transport, Atlantic cod were typically decapitated (Barrett, 1997) and thus archaeological sites can differ significantly in their bone element distribution (Orton et al., 2014). Specifically, if cod was caught locally, cranial bones may be observed in high abundance, whereas if cod was imported, postcranial bones are likely overrepresented. Bones from the pectoral girdle are anatomically close to the point of decapitation and their presence at import sites may therefore vary (Barrett, 1997; Orton et al., 2014). This variation is clear in the distribution of skeletal elements at different sites in this study (Fig. 3B). For example, cod bones found at sites in Norway and Orkney are likely to originate from local catches where cranial bones are abundant. In contrast, sites in England and in the Netherlands are characterized by a lower availability of cranial bones. Moreover, by consistently sampling the same bone element, cranial bones also offer the opportunity to easily avoid resampling the same individual.

4. Discussion

Here, we present the largest study on DNA preservation in ancient fish bones to date, assessing the effects of bone element, archaeological site, DNA extraction and sequencing library preparation protocols on library success and levels of endogenous DNA. We obtain several observations.

First, our findings imply that most fish bone elements may be

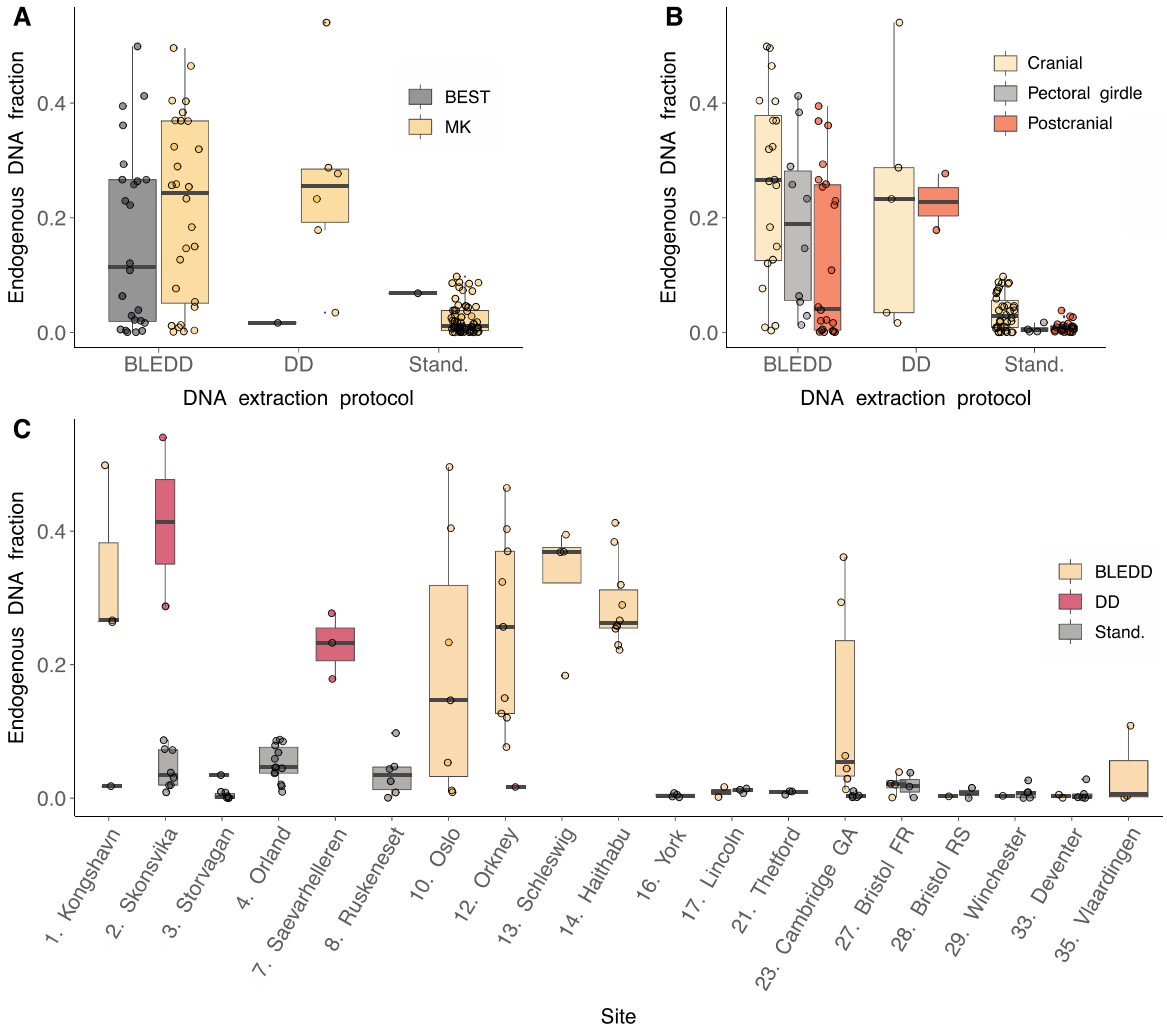

Fig. 4. Endogenous DNA fraction. (A) Endogenous DNA per extraction and library preparation protocols. Double digestion and mild bleach wash pre-treatments result in higher endogenous DNA, independently from library preparation protocols. (B) Endogenous DNA per skeletal element. No significant differences in endogenous DNA content can be observed between cranial, postcranial and pectoral girdle bones. (C) Endogenous DNA per site. Significant differences in DNA preservation can be observed between sites. DD = double digestion extraction protocol, BLEDD = bleach treatment and double digestion extraction protocol, MK = Meyer-Kircher library preparation protocol, BEST = single tube library preparation protocol. Only sites for which three or more libraries were successfully sequenced are plotted.

similarly suitable for high-throughput shotgun aDNA analyses and further archaeological inference. We did not exhaustively sample all different elements, nonetheless, when grouping bone elements into three anatomical and archaeologically relevant groups (i.e., cranial, pectoral girdle or postcranial, Box 1, Fig. 3A), we observe no significant differences in either library preparation success or endogenous DNA content amongst the different groups. This observation differs from ancient DNA results obtained from mammalian bones, where high endogenous DNA preservation is localized, either in the petrous bone (Gamba et al., 2014; Pinhasi et al., 2015) or in the dense, recently deposited circumferential lamellae of long bones (Alberti et al., 2018), with particularly poor yields from low-density spongy elements (Parker et al., 2020). This localized DNA preservation has usually been explained by the observed high density of bones or bone regions (Bollongino et al., 2008; Geigl and Grange, 2018; Kendall et al., 2018; Alberti et al., 2018) that may be more resistant to exogenous microbial colonization or taphonomic degradation (Campos et al., 2012; Gamba et al., 2014). Recently, however, it has been suggested that it is the absence of bone remodeling — rather than bone density *per se* — that helps promote DNA preservation (Kontopoulos et al., 2019; Sirak et al., 2020), following observations that the petrous bone (Kontopoulos et al., 2019), the auditory ossicle (Sirak et al., 2020) and the circumferential lamellae of long bones (Treuting et al., 2017) experience little or no bone remodeling. Interestingly, acellular fish bone is also characterized by lack of bone remodeling during growth (Kranenbarg et al., 2005; Witten and Villwock, 1997). Given the often lightweight, porous and brittle nature of fish bone, we hypothesize that such lack of bone remodeling (e.g., Kontopoulos et al., 2019; Sirak et al., 2020), may contribute to the preservation of endogenous DNA in archaeological fish samples. Further testing of this hypothesis can be achieved by comparing DNA preservation in mammal and fish bone, or remodeled and non-remodeled bones in a wider range of vertebrates, from the same archaeological site and context.

Second, depending on the down-stream computational requirements, sample sizes consisting of poor-quality DNA specimens can be increased in an economical way by avoiding pre-extraction digestion or bleach wash treatments. We observed a distinct trade-off between levels of endogenous DNA and library success when using bleach wash and pre-digestion treatments. As previously reported, bleach wash and pre-digestion treatments increase levels of endogenous DNA (e.g., Boessenkool et al., 2017; Damgaard et al., 2015; Korlević et al., 2015), yet this increase is coupled to higher failure rates during library

creation. Presumably, when samples have relatively poor DNA preservation, this DNA can be lost using pre-extracting wash steps, resulting in the failure of a sample that could otherwise yield a sequencing library with low levels of endogenous DNA. This failure results in a trade-off where the number of investigated individuals can be maximized at the cost of sequencing depth or vice-versa. Most aDNA studies, particularly if focusing on population genetics, rely on maximizing endogenous DNA content and reducing sequencing costs. For such approaches, bleach washes and/or pre-digestion treatments should be preferred. However, this trade-off can be exploited in situations where low sequencing coverage data can yield meaningful archaeological or biological information. For instance, genetic sex can be easily obtained for mammals using low numbers (e.g., <10,000) of sequencing reads, even in samples with low levels (<0.5%) of endogenous DNA (e.g., Barrett et al., 2020; Nistelberger et al., 2019; Pečnerová et al., 2017). Genetic species identification also requires few sequencing reads and is an important tool when diagnostic skeletal morphology is absent or insufficiently preserved (e.g., Grealy et al., 2016; Seersholm et al., 2018). Finally, in Atlantic cod there are several large chromosomal inversions that occur in specific spatial distributions within its geographical range (e.g., Berg et al., 2016; Berg et al., 2017). Such inversions can easily be determined using low coverage sequencing data, allowing the identification of the biological source of samples that have originated in archaeological deposits through long-distance trade (Star et al., 2017). One further aspect to consider is the availability of starting bone material. When working with fish bones of considerable smaller size than Atlantic cod, bleach washes and pre-digestion treatments may result in excessive loss of endogenous DNA. Additional testing of pre-extraction treatments for smaller fish species is needed to address this aspect.

Third, we recommend utilizing library protocols that include intermediate purification steps before adapter ligation when targeting archaeological fish bones in order to maximize the potential of successful library creation. It is advantageous to minimize hands-on-time and laboratory costs while simultaneously increasing sample throughput. For this reason, we initially implemented the single-tube (BEST) protocol (Carøe et al., 2018; Mak et al., 2017), which is an economically efficient protocol with a reduced number of purification steps compared to the Meyer-Kircher protocol (Kircher et al., 2012; Meyer and Kircher, 2010). Both protocols yield similar levels of endogenous DNA and can therefore be used to retrieve high-quality aDNA libraries from archaeological fish bone. Nonetheless, we did observe significantly increased library amplification failure rates when following BEST, which reduces the efficiency of this method in overall sample throughput. During the BEST protocol, multiple enzymatic reactions occur successively in the same tube, and we suspect it is possible that this protocol is more sensitive to inhibitors than protocols with additional purification steps such as the Meyer-Kircher protocol. The presence of inhibitors has been found to impede the efficiency of PCR-amplification of DNA from ancient fish bone before (Monroe et al., 2013). Moreover, improved success-rates for library protocols with intermediate clean-up steps compared to single-tube protocols have also been reported for degraded human bones (Young et al., 2019), suggesting that these observations are not unique to fish bone.

Finally, we conclude that a wide range of preservation and excavation conditions can yield high endogenous aDNA preservation in archaeological fish bone. We observe site-specific differences in aDNA preservation, with some sites yielding consistently high rates of library success and levels of endogenous DNA whereas others do not. These site-dependent results make it difficult to predict specific factors underlying sufficient aDNA preservation, as samples from each site are associated with a wide range of different, potentially unknown, *pre-* and *post-*excavation taphonomic processes. However, our results confirm that cave sites typically offer ideal conditions for DNA preservation (Bollongino et al., 2008; Hardy et al., 1995), most likely thanks to stable low temperatures and lack of precipitation (Hedges and Millard, 1995). Here, we report on one of the oldest WGS results for archaeological fish bone (see also Kirch et al., 2020) from the cave site of Sævarhelleren (site 7, Bergsvik et al., 2016), which is one of the sites with better DNA preservation despite being up to 8500 years old. In addition to this, we have obtained excellent DNA of bones obtained from dry shell middens (e.g., Orkney Quoygrew, site 12, Harland and Barrett, 2012), as well as bones from waterlogged sediments that were excavated decades ago (e.g., Haithabu Harbour, site 14, Heinrich, 2006).

So far, only limited number of fish aDNA studies have reported on the potential of aDNA obtained from fish bone, despite its abundance in the archaeological record, and the environmental, as well as past and present economic importance of fish (Barrett, 2019; Oosting et al., 2019). Especially whole genome HTS approaches (e.g., Star et al., 2017) from fish remains are rare. Here we show that, despite high variability in DNA preservation across archaeological sites, high endogenous aDNA can consistently be recovered from archaeological Atlantic cod specimens from a range of different locations. Overall, we obtained successful sequencing libraries from 50% of all samples, retrieving more than 20% endogenous DNA from 40% of sites. Atlantic cod is a comparatively large fish, and samples were obtained from sites at northern latitudes from mild to cold climates. These characteristics likely improve the potential for aDNA preservation (Willerslev and Cooper, 2005). Nonetheless, studies on smaller species such as Pacific herring (e.g., Speller et al., 2012) or stickleback (Kirch et al., 2020), or samples obtained from sites in warmer climates (e.g., Grealy et al., 2016) have reported the successful retrieval of aDNA from fish bone. The improved availability of genome assemblies for an increased number of fish species remains important for the successful application of HTS approaches on a greater taxonomic scale. Such HTS studies covering a wider range of fish species and climates, possibly in combination with novel laboratory protocols (e.g. Troll et al., 2019; Gansauge et al., 2020), will further establish archaeological fish bone as a material suitable for ancient DNA analyses.

Data availability

All ancient read data are available at the European Nucleotide Archive, www.ebi.ac.uk/ena, (accession no. PRJEB37681).

Author contributions

B.S., G.F. and J.H.B designed research; O.K., A.H.P., A.T.G. and G.F. carried out laboratory work. A.C. and G.F. analyzed data. A.K.H., I.Y., I. J., S.W., G.F.B., J.H., R.N., D.O., B.C., R.B. and J.H.B. provided samples and archaeological context information. R.B. selected UK specimens. B. S. and G.F. wrote the manuscript with input from all authors. B.S., J.H.B. and S.B. provided funding and consumables.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We thank C. Amundsen for providing the Kongshavn and Skonsvika bone samples, M. Skage, S. Kollias and A. Tooming-Klunderud at the Norwegian Sequencing Centre for sequencing and processing of samples, and K. Dean for comments on the statistical analyses. This work was supported by Research Council of Norway Project “Catching the Past: Discovering the legacy of Atlantic cod exploitation using ancient DNA” (262777) and Leverhulme Trust Project “Northern Journeys: Reimagining the Medieval Revolution and its Aftermath” (MRF-2013-065). Analyses were performed on the Abel Cluster at the University of Oslo and the Norwegian metacentre for High Performance Computing (NOTUR), operated by the Department for Research Computing at the University of Oslo IT-department (USIT).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jas.2020.105317.

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